167 Immunologic Tests

DEBORAH A. WILSON

The diagnosis and management of diseases affecting the musculoskeletal system may be facilitated by using the results of one or more immunologic tests. In using such tests for assistance in diagnosis, however, the results of laboratory studies should be considered supportive or confirmatory of the clinical presentation based on an accurate, complete history and physical examination. In some situations, anticipated positive laboratory findings may be absent despite evidence of disease. Some of the tests are nonspecific and results may be present or abnormal because of other diseases, the patient's age, or the genetic makeup of the patient. The laboratory studies to be discussed are those most frequently used in the diagnosis and management of patients with diseases affecting the musculoskeletal system.

Acute Phase Reactants

A number of components of plasma are increased within the first few days following tissue destruction by inflammation, infection, or trauma. These "acute phase reactants" may be proteins, glycoproteins, lipoproteins, or other substances and are produced predominantly in the liver. If the inflammatory insult is brief, the levels of acute phase reactants return to normal within days or weeks; however, they may remain elevated in chronic infections or inflammatory conditions. The most commonly studied acute phase proteins in humans include C-reactive protein, haptoglobin, fibrinogen, serum amyloid A protein (SA-A); alpha-1 proteins including alpha-1 antitrypsin, ceruloplasmin; and components of the complement cascade, especially C3. The tests used most frequently in clinical practice for the acute phase response are the erythrocyte sedimentation rate and measurement of C-reactive protein.

Erythrocyte Sedimentation Rate

Definition

The erythrocyte sedimentation rate (ESR) is a nonspecific measurement of the acute phase response. It is the rate of fall of erythrocytes in a column of blood in 1 hour. The generally accepted upper limit of normal value for young adults by the Westergren method is 15 mm per hour for males and 20 mm per hour for females. In the elderly the normal range is slightly higher.

Technique

The ESR is a simple test that can be performed in a physician's office. Two methods most commonly used are the Wintrobe method and the Westergren method. The Westergren method has been declared the superior method by the International Committee for Standardization in Hematology.

The Westergren method requires collecting 2 ml of venous blood into a tube containing 0.5 ml of sodium citrate. It should be stored no longer than 2 hours at room temperature or 6 hours at 4°C. The blood is drawn into a Westergren–Katz tube to the 200 mm mark. The tube is placed in a rack in a strictly vertical position for 1 hour at room temperature, at which time the distance from the lowest point of the surface meniscus to the upper limit of the red cell sediment is measured. The distance of fall of erythrocytes, expressed as millimeters in 1 hour, is the ESR. Although the results may be affected slightly by anemia, the International Committee for Standardization in Hematology does not recommend that any correction factor be used in interpreting the results.

The Wintrobe method is performed similarly except that the Wintrobe tube is smaller in diameter than the Westergren tube and only 100 mm long. Anticoagulated blood without extra diluent is drawn into the tube, and the rate of fall of erythrocytes is measured in millimeters after 1 hour. Correction for anemia, since the tube is short, may be necessary because a low hematocrit can cause the ESR to appear to be greater. The shorter column also makes this method less sensitive than the Westergren method because the maximal possible abnormal value is lower.

A newer technique, the zeta sedimentation ratio, is another method of indirectly measuring the acute phase response. For this test, blood is drawn into 75 × 2 mm capillary tubes, placed vertically in a special centrifuge, the Zetafuge, and the tubes are spun at 400 rpm for four 45-second periods. The tubes are rotated 180 degrees on the vertical axis after each spin. The aggregation of red cells occurs in a downward zigzag path, affected both by centrifugal force and gravity. The test requires approximately 5 minutes. The degree of compaction of the erythrocytes is expressed as the zetacrit by dividing the height of the red cell sediment by the height of the column of blood. A hematocrit is also done, and the ratio of the hematocrit to the zetacrit is the zeta sedimentation ratio (ZSR). The ZSR is thus unaffected by anemia, and values in men and women are similar. Good correlation of the ZSR with simultaneously run ESRs has been shown. The ranges of values obtained in a study of

normal patients comparing the Westergren, Wintrobe, and zeta methods are shown in Table 167.1.

Basic Science

The rate of fall of erythrocytes in a column of blood is influenced by the extent to which erythrocytes are aggregated. The three major factors that influence erythrocyte aggregation are the surface free energy of the red cells, the surface charge of the red cells, and the dielectric constant of the medium in which the cells are suspended. The surface free energy of red cells acts as an attracting force due to van der Waals' forces, whereas the negatively charged surface of the red cells acts to repel cells from each other. A positively charged cloud surrounds the negatively charged cells, and these two charges result in the zeta potential. The dielectric constant of the medium acts to dissipate the charges. The dielectric constant is affected by the concentration and symmetry of plasma proteins. Asymmetrical plasma proteins cause an increase in the dielectric constant by polarizing red cells, thereby diminishing the repulsive forces of the red cells and facilitating agglutination. The rate of erythrocyte sedimentation is directly proportional to the amount of erythrocyte aggregation and the size of the aggregates. The most asymmetric acute phase protein is fibrinogen, and it has the greatest effect on the ESR. Other acute phase proteins, especially alpha and gamma globulins, may also enhance aggregation when present in large concentrations.

The ESR is influenced by several factors other than plasma proteins. Alterations in the shape of red cells may prevent aggregation, resulting in a normal ESR regardless of the presence of acute phase reactants. Anemia can accelerate the ESR and polycythemia can retard it. Heparin can increase the ESR, as can some intramuscular injections. Ingestion of food also causes fluctuation of the ESR.

Clinical Significance

Although the level of the ESR generally correlates with the severity of inflammation or tissue injury, it is a nonspecific test that can be influenced by other factors. In acute severe bacterial or viral infections or other causes of acute inflammation or tissue injury, such as myocardial infarction or trauma, the ESR should rise initially and then fall with recovery. In chronic infections or inflammatory conditions such as osteomyelitis, rheumatoid arthritis, or vasculitis it may also fluctuate with exacerbations and remissions. Since high levels of immunoglobulins also cause an elevation of the ESR, lymphoproliferative disorders such as multiple myeloma and Waldenström's macroglobulinemia should also be considered in patients with a markedly elevated ESR. However, some patients with obvious inflammatory disease may have a normal ESR. Elevation of the ESR has also been reported in noninflammatory conditions such as thyroid disease and late in pregnancy.

Because the ESR is a nonspecific test that can be used to screen for inflammatory or tissue-damaging conditions, it is necessary for the physician to know how to respond to an abnormal value. When the ESR is mildly elevated without an obvious source, further evaluation should be limited, since studies have shown that the degree of elevation of the ESR is of little help in differential diagnosis. In contrast, patients in whom the ESR is greater than 100 mm/hr by the Westergren method often have serious disease. The most common causes of a markedly elevated ESR are hematologic and nonhematologic malignancy; infection; connective tissue disease, especially rheumatoid arthritis and vasculitis, including giant cell arteritis; and renal disease. Most of these diagnoses can be made by performing a complete history and physical examination as well as a complete blood count, routine urinalysis, and chest roentgenogram. Additional studies, including serum protein electrophoresis, bone marrow examination, and renal function tests, may be of diagnostic value in some patients.

Polymyalgia rheumatica, a common disease of the elderly characterized by painful stiffness of the pectoral and pelvic girdles and temporal (giant cell) arteritis, deserves special mention. These signs and symptoms can occur simultaneously or separately and are nearly always associated with an ESR greater than 50 mm/hr by the Westergren method.

Some of the limitations of the ESR have been described. A major criticism is that it is not a specific measure of acute phase proteins, and thus of the acute inflammatory response, and that methods for quantification of many of the acute phase proteins are available. However, the simplicity and wide availability of the ESR favor its value in clinical medicine.

Table 167.1
Comparison of Normal Values of Erythrocyte Sedimentation by Westergren, Wintrobe, and Zeta Methods

Method		Results
Westergren	Males	0–10 mm/hr
	Females	3–25 mm/hr
Wintrobe	Males	1–11 mm/hr
	Females	6–24 mm/hr
Zeta	Males	46–52%
	Females	46–54%

Source: Modified from Bucher, WC, Gall, EP, Woodworth, R. Am J Clin Pathol 1975;64:613–17. The results indicate the range of the mean value ± 1 *SD*. 104 normal patients (49 males, 55 females), age 21 to 60 years, were studied.

References

*Bedell SE, Bush BT. Erythrocyte sedimentation rate. From folklore to facts. Am J Med 1985;78:1001–09.

Bucher WC, Gall EP, Woodworth R. Zeta sedimentation ratio in rheumatic disease: comparison of the zeta sedimentation ratio with the Wintrobe and Westergren sedimentation rates. Am J Clin Pathol 1975;64:613–17.

Bull BS, Brailsford JD. The zeta sedimentation ratio. Blood 1972;40:550–59.

Ford MJ, Innes JA, Parrish FM, et al. The significance of gross elevations of the erythrocyte sedimentation rate in a general medical unit. Eur J Clin Invest 1979;9:191–94.

International Committee for Standardization in Hematology. Reference method for the erythrocyte sedimentation rate (ESR) test on human blood. Br J Haematol 1973;24:671–73.

Kushner I. Erythrocyte sedimentation rate and the acute phase reactants. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of Rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;719–727.

Sox HC Jr, Liang, MH. The erythrocyte sedimentation rate: guidelines for rational use. Ann Intern Med 1986;104:515–23.

Zacharski LR, Kyle RA. Significance of extreme elevation of erythrocyte sedimentation rate. JAMA 1967;202:116–18.

C-Reactive Protein

Definition

C-reactive protein (CRP) is a nonglycosylated protein produced by human hepatocytes in response to infection, inflammation, or tissue damage. It is composed of five identical noncovalently linked subunits that form a symmetrical pentagonal structure with a molecular weight of 105,000 daltons. CRP was originally named for its ability to precipitate somatic C-polysaccharide of pneumococci; however, other methods are now used to detect it.

CRP is present at very low levels in the normal population. Levels less than 1 mg/dl are considered insignificant, levels from 1 to 10 mg/dl are considered moderately elevated, and levels greater than 10 mg/dl are markedly elevated.

Technique

Before quantitative methods for CRP were developed, CRP was reported as "present" or "absent." Several methods are now available for its determination; the most common are radial immunodiffusion, electroimmunodiffusion, and immunonephelometry. These methods use monospecific anti-CRP antiserum or antibodies that bind CRP as antigen.

In the radial immunodiffusion procedure, antiserum with anti-CRP activity is incorporated into agarose gel coated onto a glass plate. Wells are made in the agarose, and a small amount of human serum is placed in the well. The plate is incubated for approximately 48 hours. The antigen (CRP) from the human serum diffuses radially into the agarose, forming a precipitin ring. The diameter of the ring is measured and compared to the size of rings of simultaneously or previously determined control sera in which the amount of CRP is known (Figure 167.1).

The electroimmunodiffusion method also uses antiserum to human CRP incorporated into agarose on a glass plate. Human serum or standards are placed into wells in the agarose, and an electrical current is applied across the plate for 45 minutes. The electrical current rapidly draws the antigen from the well, and a cone-shaped peak is formed as the antigen precipitates with antibody. The distance from the sample origin to the top of the precipitin peak is measured and compared to standards containing known quantities of CRP. This test requires approximately 2 hours.

Nephelometry is the measurement of turbidity or cloudiness. In immunonephelometric assays, the degree of light scatter created by the formation of antigen–antibody complexes is detected. An instrument with a light source of various wavelengths (such as a laser source) directs the light beam through a tube containing test serum and optically clear monospecific antibodies in an optically clear solution, in this instance anti-CRP antibodies. The intensity of light scattered by the precipitating complexes is measured by a light-detection system and converted into concentration units. The rate nephelometry method is currently the superior nephelometry method because it measures the light scatter rapidly several times during the ascending phase of the antigen–antibody reaction before equivalence is reached. If the CRP level is extremely high, the patient's serum must be diluted.

The immunodiffusion techniques are very sensitive, detecting levels of CRP as low as 0.2 mg/dl. The nephelometric method can detect levels as low as 0.6 mg/dl, very adequate for clinical use.

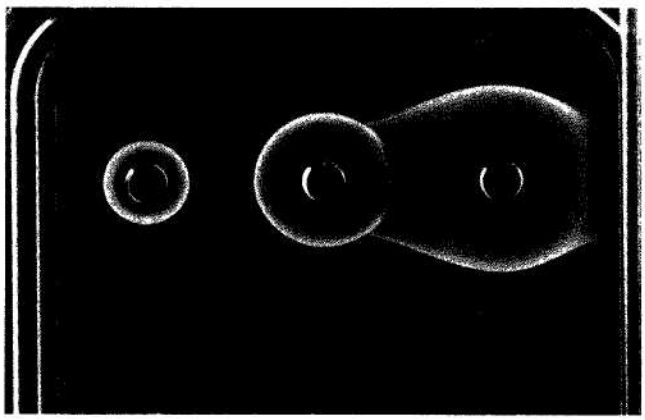

Figure 167.1
Radial immunodiffusion. The patient's serum is placed in the wells cut out of the agarose gel. The agarose gel contains antibodies to the test antigen (CRP in this case). The antigen diffuses into the agarose and forms precipitin rings. The diameter of the rings is directly proportional to the amount of antigen in the serum and is quantified by comparing the size of the ring to that of simultaneously tested standards.

Basic Science

The precise physiologic role of CRP is not known; however, the results of many in vitro studies suggest that it is of pathophysiologic importance. CRP binds to a variety of ligands, especially to phosphorylcholine. It can also bind to a variety of proteins, lipids, and polysaccharides, resulting in precipitation or agglutination. Subsequent activation of the classic complement pathway has been demonstrated in vitro. CRP binds to plasma membranes of damaged cells but not to intact living cells. CRP has also been shown to reduce the toxicity or pathogenicity of microorganisms, perhaps by binding to them or initiating complement activation and phagocytosis. Other possible roles of CRP include the enhancement of resolution and repair of damaged tissue by initiating complement activation, opsonization and phagocytosis, detoxification or facilitation of clearance of foreign materials from the circulation, and initiation of the inflammatory response.

The magnitude of CRP elevation correlates well with the severity of disease. Following the onset of the inflammatory stimulus, CRP levels begin to rise within a few hours and peak within 48 hours. Depending on the type and chronicity of the stimulus or treatment, levels may fall rapidly or remain elevated.

Clinical Significance

Severe bacterial infection is usually the most potent stimulator of CRP production. Many rheumatic diseases that cause acute and chronic inflammation are also associated with CRP elevation during periods of disease activity. Pa-

tients with active arthritis including adult and juvenile rheumatoid arthritis, Reiter's syndrome, psoriatic arthritis, ankylosing spondylitis, acute and chronic gout, and rheumatic fever have elevated CRP levels. CRP levels are increased in most patients with vasculitis such as polyarteritis nodosa, Wegener's granulomatosis, giant cell arteritis, polymyalgia rheumatica, and both cutaneous and systemic necrotising vasculitis. In contrast, systemic lupus erythematosus (SLE), polymyositis, and scleroderma usually cause only modest CRP elevations.

The CRP level can thus be useful as an unequivocal sign of tissue damage. Serial levels can be used for monitoring changes in disease activity and responses to medical treatment. CRP levels may also be useful in diseases that are not usually associated with high CRP levels, such as SLE, in which cases marked elevations may indicate infection or other complications.

Although the CRP response is nonspecific and cannot be used alone in differential diagnosis, it is a valuable test and has advantages over the ESR. It is a measure only of tissue damage and is not affected by immunoglobulin levels. Levels of CRP rise and fall more rapidly and over a broad range with changes in the clinical condition. Measurement of CRP is sensitive and reproducible. The level does not have diurnal variation, and it is not affected by age, sex, or hematocrit.

References

Gill CW, Bush WS, Burleigh WM, et al. An evaluation of a C-reactive protein assay using a rate immunonephelometric procedure. Am J Clin Pathol 1981;75:50–55.

Kushner I. Erythroid sedimentation rate and the acute phase reactants. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;719–727.

Morley JJ, Kushner I. Serum C-reactive protein levels in disease. Ann NY Acad Sci 1982;389:406–18.

Pepys MB, Baltz ML. Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. Adv Immunol 1983;34:141–212.

Stites DP, Rodgers RPC. Clinical laboratory methods for detection of antigens and antibodies. In: Stites DP, Stobo JD, Fudenberg HH, Wells JV, eds. Basic and clinical immunology. 6th ed. Norwalk and Los Altos: Appleton and Lange 1987;241–84.

Immunoglobulins and Cryoglobulins

Definition

Immunoglobulins (Ig) are a heterogeneous group of complex protein molecules that function as antibodies to a wide variety of antigens. Each immunoglobulin subunit is composed of four polypeptide chains, two heavy chains and two light chains. The heavy chains are joined to each other, and the light chains are held to the heavy chains by disulfide bonds (Figure 167.2). The five major immunoglobulin classes in humans are IgG, IgM, IgA, IgD, and IgE. These classes differ in the structure of the heavy chain. IgG and IgA are further divided into subclasses IgG_1, IgG_2, IgG_3, IgG_4, and IgA_1 and IgA_2. There are two types of light chains, kappa and lambda, which are not class specific. In humans, kappa chains predominate in frequency over lambda chains. Each immunoglobulin chain has a constant region and a variable region. The constant region is similar within each class; certain positions of the variable region function as the antigen-binding site. The normal ranges for immunoglobulin levels are shown in Table 167.2.

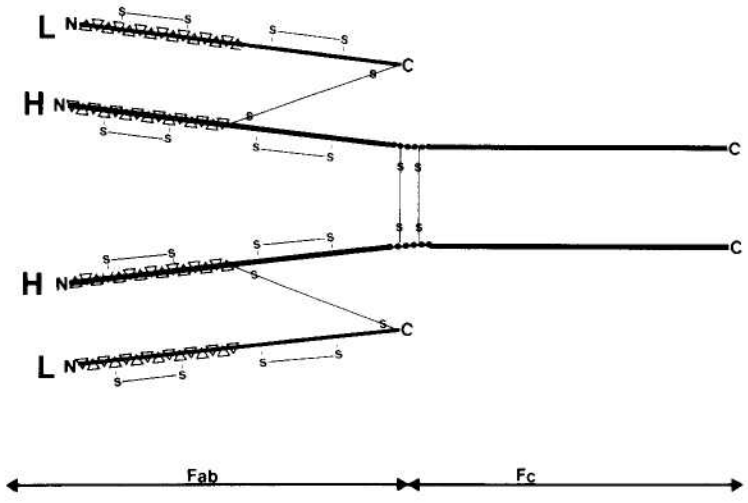

Figure 167.2
Simplified model of an immunoglobulin. The heavy lines represent heavy *(H)* and light *(L)* chains; the light lines *(S—S)* represent disulfide bonds. The variable regions of H and L chains are indicated by ∇Δ and the hinge region by .... *C* indicates carboxy terminus, and *N* indicates amino terminus.

Table 167.2
Immunoglobulin Levels in Normal Adults

Immunoglobulin class	Serum level (mg/dl)[a]
IgG	639–1349
IgM	56–352
IgA	70–312

[a]Values indicate normal range when determined by nephelometry. IgD and IgE levels are minimal and are not determined for routine screening.

Cryoglobulins are immunoglobulins that precipitate on exposure to cold. Cryoimmunoglobulins G, M, and A have been identified. The normal level is less than 0.2% as a "cryocrit," or less than 0.1 mg per deciliter of serum.

Technique

Serum protein electrophoresis may be used as a screen for the total serum immunoglobulin level; however, other methods are more specific and quantitative. It is most appropriately used to screen for paraproteins such as occur in multiple myeloma or Waldenström's macroglobulinemia. To perform this test, serum is applied to a strip of paper, agarose gel, or cellulose acetate and electrophoresed for approximately 90 minutes. Five major bands of protein are separated in normal human serum on the basis of their charge: albumin, alpha$_1$-globulins, alpha$_2$-globulins, beta-globulins, and gamma-globulins. The bands are stained to allow a densitometer to quantify the protein concentration of each band. The results are expressed as shown in Figure 167.3. A paraprotein can thus be detected as an abnormal peak, usually in the gamma or beta region.

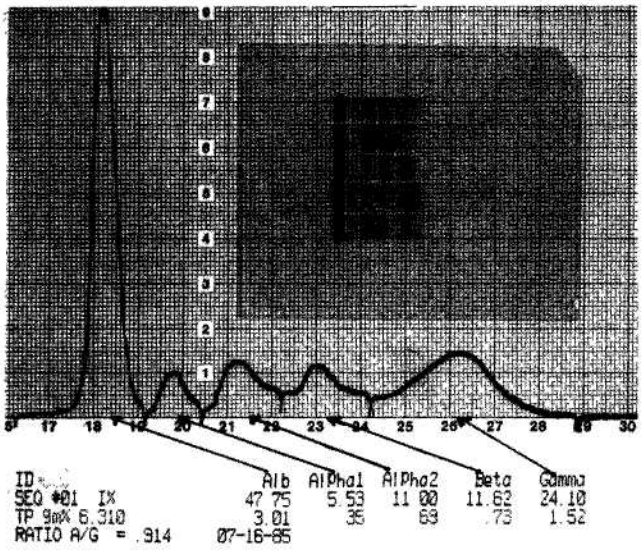

Figure 167.3
Serum protein electrophoresis. In the inset are shown five separate electrophoresis samples with five bands each, some of which are very light. The heavy bands are albumin followed by the different globulins. The bands from one of the electrophoreses have been converted to peaks by densitometer scanning. Below the identification of each peak is shown the percentage of the total protein and the absolute amount of protein per peak (mg/100 ml serum).

Further identification and approximate quantification of abnormal immunoglobulins or light chains can be obtained by immunoelectrophoresis (Figure 167.4). This is a double immunodiffusion technique. Serum (or other biologic fluid such as urine, CSF) is placed in a well on a glass slide coated with agarose or cellulose acetate, and electrophoresed to separate the proteins according to their charge. A trough is cut in the agarose parallel to the axis of the electrophoresed proteins into which is placed monospecific antibodies against IgG, IgM, IgA, or kappa or lambda light chains. The slide is incubated for 18 to 24 hours to allow the antibodies to diffuse from the trough into the agarose, forming precipitin arcs with their respective antigens. The relative size of the precipitin arcs is proportional to the quantity of immunoglobulin or light chains.

Immunofixation electrophoresis is also used clinically to identify monoclonal proteins in serum, urine, or CSF. It is a two-stage procedure in which the serum or other body fluid is electrophoresed in gel to separate the proteins, followed by immunoprecipitation of the specimen proteins and control proteins for comparison with monospecific antisera. The gel is then washed to remove nonprecipitated proteins, followed by application of a protein stain to visualize the antigen-antibody complexes. This method has the advantage of more easily identifying IgM and IgA monoclonal proteins that may be masked by IgG. Reagents for IgD and IgE monoclonal proteins are also available. This

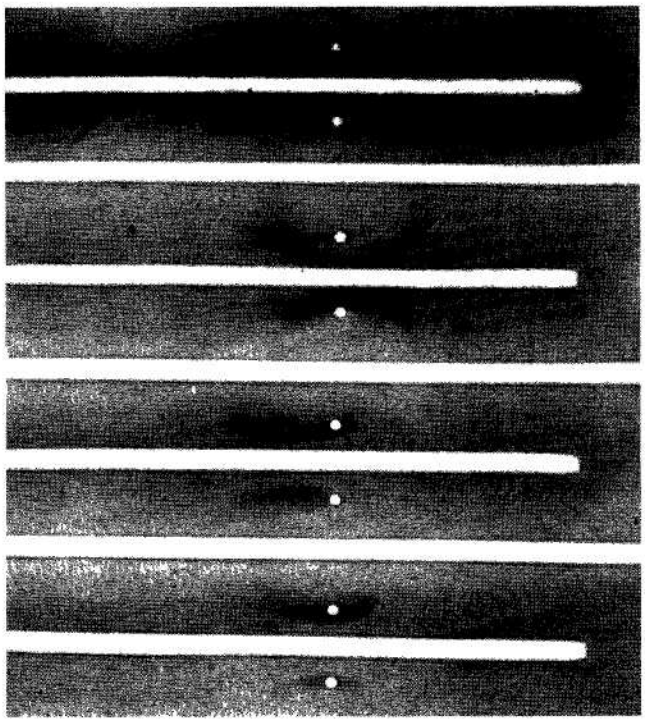

Figure 167.4
Immunoelectrophoresis. Serum from a normal control was placed in the top well and the patient's undiluted serum was placed in wells 2, 3, 5, and 7, reading down. Wells 4, 6, and 8 contained a 1:5 dilution of the patient's serum. The top trough contained antibodies to whole human serum. The third trough contained antibodies to human IgG; the fifth trough, antibodies to human IgA; and the seventh trough, antibodies to human IgM. The varied positions of the precipitin bands indicate the differences in migration of the proteins in an electric field.

test can be completed in 90 minutes, which is another definite advantage.

Nephelometric or radial immunodiffusion assays are the tests of choice for further quantification of immunoglobulins G, M, and A. In the radial immunodiffusion technique, antibodies to Fc or heavy chain determinants of immunoglobulins G, M, or A are incorporated into the agarose, and serum is placed in a well. The amount of Ig in the patient's serum is proportional to the diameter of the precipitin ring formed by complexed Ig–anti-Ig. Nephelometry also requires the use of monospecific antibodies to immunoglobulins. The antibodies, the patient's serum, and a buffer solution are added to a glass tube and a light source is directed through the tube. The degree of light scattered by Ig–anti-Ig complexes is detected by a light detector and read as optical density. This reading is converted to concentration units of protein.

To isolate cryoglobulins, special care must be taken in drawing the blood specimen. Ideally, blood should be drawn into a warm syringe without anticoagulant, transferred to a warmed tube, transported to the laboratory in a cup containing water at 37°C, allowed to clot in a 37°C waterbath, and centrifuged at 37°C. Practically, as many of these conditions as possible should be met. The blood may be drawn into a tube at room temperature, placed in a cup of warm water or one's pocket to be kept warm by body temperature, and transported quickly to the laboratory to be placed in a 37°C waterbath. After the blood has clotted, it can be centrifuged at room temperature if the tube is placed in cups containing 37°C water. The serum is removed and placed in a conical centrifuge tube, covered, and stored at 4°C. Because some cryoglobulins precipitate slowly, the serum should be stored for 7 days, with daily observation. Most cryoglobulins will appear in 24 to 72 hours. To assure that a precipitate is a cryoprecipitate, it should be rewarmed to 37°C and observed to resolubilize. After recooling, the serum is centrifuged, and the material that precipitates is considered to be cryoglobulin. This can be quantified by decanting the serum, resuspending the precipitate in saline, and determining the protein content spectrophotometrically or colorimetrically, such as by the Lowry method. Another method of quantification is the "cryocrit." One recommended procedure entails placing a known quantity of water in a second tube (identical to that which contains the precipitate) to the same level as the precipitate, and thus determine the volume of the precipitate. The percentage of the total volume of serum occupied by the precipitate can be calculated as the cryocrit. Once the cryoglobulin is quantified, it can be resuspended for analysis by immunoelectrophoresis. Radial immunodiffusion is less reliable because of the poor diffusing capability of some complex cryoproteins. Rheumatoid factor activity of the cryoprecipitate may also be determined.

Basic Science

The primary function of immunoglobulins is to act as antibodies that combine with and facilitate the removal of foreign antigens from the system. The rate of antibody production is thus influenced by the degree of antigenic stimulation. Antibodies are produced by plasma cells that mature from B lymphocytes. The amount and class of antibody produced are influenced by the effect of many different types of regulatory T lymphocytes and by the type of antigen. The structure of immunoglobulins and the differentiation of B cells to produce different classes of antibodies are controlled genetically.

Numerous abnormalities of immunoglobulin production can occur, including congenital or acquired deficiencies of one or all of the immunoglobulin classes, malignant proliferation of a single clone of immunoglobulin-producing cells, benign monoclonal gammopathies that may ultimately develop into the production of malignant paraproteins, or polyclonal gammopathies that are usually associated with underlying infectious or inflammatory diseases.

One of the phenomena associated with overproduction of certain immunoglobulins is cryoglobulinemia. The cryoglobulins have been classified into three types: Type 1, *monoclonal cryoglobulins*, usually of the IgM class and less often of the IgG and IgA classes; Type 2, *mixed monoclonal cryoglobulins* containing monoclonal IgM in most cases and, infrequently, monoclonal IgG or IgA that have rheumatoid factor activity against polyclonal IgG; and Type 3, *mixed polyclonal cryoglobulins* that are composed of one or more classes of polyclonal immunoglobulin, usually IgM, with antibody activity against polyclonal IgG. Other proteins such as complement or lipoproteins can also be complexed with Type 3 cryoglobulins.

It is important to be aware of the possible presence of cryoglobulins when evaluating serum immunoglobulins. Failure to detect a paraprotein or immunoglobulin with cryoprecipitating properties can result from allowing blood to clot at room temperature or lower, because the cryoproteins may precipitate with the clot and be removed from the serum with centrifugation. If one's suspicion is high for cryoglobulins, the serum to be used for protein electrophoresis or immunoelectrophoresis should be kept warm. Similarly, the sedimentation rate at room temperature may appear falsely low due to gelling of serum containing cryoproteins, whereas it would be elevated if performed at 37°C.

The temperature at which cryoglobulins precipitate is variable and can range from 10 to 36°C. The reason for cryoprecipitation is not yet known but is apparently influenced more by concentration and molecular composition than by size, shape, or charge of the complex.

Clinical Significance

There are relatively few clinical conditions affecting the musculoskeletal system for which qualitative or quantitative evaluation of serum immunoglobulins is of diagnostic significance, but some of these conditions can be serious and warrant the use of these tests for screening when the clinical findings are compatible with signs and symptoms of these diseases. Multiple myeloma patients may present with an arthropathy that can mimic rheumatoid arthritis but is actually caused by amyloid deposition in the synovium and around the joints. Both congenital and acquired hypogammaglobulinemia can be associated with rheumatoid-like arthritis in addition to recurrent infections. Selective IgA deficiency is common, occurring in one out of 600 to 800 individuals, and may be associated with a variety of autoimmune disorders, especially rheumatoid arthritis and SLE. A hyperglobulinemic state may occur in Sjögren's syndrome, multiple myeloma, or without known underlying etiology. This results in the formation of IgG–IgG complexes that cause hyperviscosity and vascular injury, especially in the lower extremities, and should be distinguished from other vasculitides. Polyclonal elevation of immunoglobulins is seen frequently in inflammatory disorders such

as SLE, mixed connective tissue disease, rheumatoid arthritis, and systemic vasculitis. Other laboratory tests, in addition to careful clinical observations, are usually more appropriate for diagnosis and evaluation of disease activity in these latter conditions.

Cryoglobulinemia may be found in a wide variety of clinical conditions. In many patients no underlying disease can be found. Most of these patients have Type 3 cryoglobulins and the disease is called essential mixed (polyclonal) cryoglobulinemia. Some authors have hypothesized that the polyclonal immunoglobulins, usually IgM, have antibody activity against polyclonal IgG that is specific for an unknown antigen. Recent studies have shown, however, that many patients with essential mixed cryoglobulinemia have hepatitis B surface antigen or antibody in the cryoprecipitate. Other conditions commonly associated with Type 3 cryoglobulins are autoimmune diseases such as SLE, Sjögren's syndrome, rheumatoid arthritis, and systemic vasculitis. Chronic infections such as subacute bacterial endocarditis and Lyme disease and viral infections may also be associated with circulating Type 3 cryoglobulins. Patients with Type 3 cryoproteins commonly have vasculitic purpura, articular symptoms, glomerulitis, hepatic involvement, and, less frequently, peripheral sensory and motor neuropathy.

Type 1 cryoglobulinemia is most often associated with multiple myeloma or Waldenström's macroglobulinemia. These patients often have symptoms associated with hyperviscosity (e.g., digital necrosis, leg ulcers, or hemorrhage) but may also have renal involvement. Raynaud's phenomenon, which is episodic vasospasm of the digits on exposure to cold, is common to all three types of cryoglobulinemia.

Type 2 cryoglobulinemia is most often associated with lymphoproliferative malignancy, multiple myeloma, or Waldenström's macroglobulinemia, but can also occur in Sjögren's syndrome and rheumatoid arthritis. The symptoms associated with Type 2 cryoglobulins include both those due to hyperviscosity and those associated with vasculitic phenomena, as in Type 3 cryoglobulinemia.

References

Brouet J-C, Clauvel J-P, Danon F, et al. Biologic and clinical significance of cryoglobulins: a report of 86 cases. Am J Med 1974;57:775–88.

Gorevic PD, Hannah JK, Levo Y, et al. Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. Am J Med 1980;69:287–308.

Koopman WJ, Griffin JA. B lymphocytes. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;169–197.

Lightfoot RW Jr. Cryoglobulinemia and other dysproteinemias. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989; 1337–50.

Stites DP, Rodgers RPC. Clinical laboratory methods for detection of antigens and antibodies. In: Stites DP, Stobo JD, Fudenberg HH, Wells JV, eds. Basic and clinical immunology. 6th ed. Norwalk and Los Altos: Appleton and Lange, 1987;241–284.

Talal N. Sjögren's syndrome and connective tissue diseases associated with other immunologic disorders. In: McCarty DJ, ed. Arthritis and allied conditions. 11th ed. Philadelphia: Lea & Febiger, 1989;1197–1213.

Rheumatoid Factor

Definition

Rheumatoid factors are autoantibodies that bind a variety of antigenic determinants of the Fc portion of immunoglobulin G (IgG) antibodies. The autoantibodies may be of the IgM, IgG, IgA, and IgE classes. They were first detected in the sera of rheumatoid arthritis patients by Waaler, and later by Rose in the 1940s, but have subsequently been found in the sera of patients with a variety of other diseases. Normal values of serum rheumatoid factors have not been defined; however, high titers of IgM and IgG rheumatoid factors are usually indicative of active inflammatory or infectious disease.

Technique

The most commonly used tests for IgM rheumatoid factor have been the latex and bentonite agglutination tests and the Rose–Waaler or sheep cell agglutination test. For the latex and bentonite tests, latex or bentonite particles are coated with human IgG that acts as antigen for IgM rheumatoid factor. IgM molecules, including IgM rheumatoid factors, are pentavalent, and when they combine with the IgG on the particles, cross-linking occurs and causes the particles to agglutinate visibly. Serial dilutions of patient serum are added to the IgG-coated particles, and the highest dilution of serum that causes agglutination is the "rheumatoid factor titer."

In the Rose–Waaler test, sheep erythrocytes are coated with rabbit IgG. Serial dilutions of human serum are added to aliquots of Ig-coated sheep cells that are visibly agglutinated by the presence of IgM rheumatoid factor.

Two other very sensitive methods that can be used to detect IgM rheumatoid factor are the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). For the RIA, plastic tubes or microtiter wells are coated with human IgG. The test serum is added to the tubes or wells and incubated to allow rheumatoid factor to bind to the IgG. The tubes or wells are then washed, leaving the bound rheumatoid factor. A secondary radiolabeled anti-human Ig antibody is then added. After washing to remove unbound secondary antibody, the radioactivity in the tubes or wells is determined and the amount of rheumatoid factor present can be quantified. The ELISA method is similar except that the secondary anti-Ig antibody is coupled to an enzyme. After washing, a substrate solution is added and changes color when altered by the enzyme. The rheumatoid factor level is quantified by determining the optical density of the substrate solution spectrophotometrically.

Rate nephelometry is also used frequently for measurement of IgM rheumatoid factor. Aggregated IgG is used as antigen and the rate of increase of light scattered from particles suspended in solution as a result of complexes

formed during the reaction of IgM rheumatoid factor with IgG is measured.

The results of the RIA, ELISA, and nephelometry tests can be reported as International Units per milliliter (IU/ml) of patient serum since a World Health Organization Reference Preparation of Rheumatoid Factor exists. There is much variability among laboratories and commercial preparations of these tests, however, so it is often not possible to make direct comparisons of normal and abnormal values between tests.

The quantification of IgG rheumatoid factor is more difficult because it does not have agglutinating properties and because it tends to self-associate in dimers or trimers. It can be detected by an ultracentrifuge method or by recently developed RIA and ELISA methods. RIA, ELISA, and other methods can also be used to quantify IgA and IgE rheumatoid factors by using Ig class-specific secondary antibodies.

Basic Science

The production of antibodies that combine with self-IgG as antigen is an autoimmune phenomenon for which the etiology is not precisely known although several mechanisms have been proposed. It is possible that the IgG is altered in some way by changes in physicochemical conditions or by exposure of otherwise hidden antigenic determinants when immune complexes are formed during antigen–antibody responses. The Fc portion of IgG may also contain antigenic determinants that are similar to other exogenous or self-antigens, resulting in cross reactivity. Sensitization may occur during pregnancy. Aberrations of the immunoregulatory status of the individual may lead to rheumatoid factor production, such as changes in T cell regulation of B cells, or loss of B and/or T cell tolerance to IgG. Foreign antigens or mitogens may cause polyclonal B cell activation, resulting in the production of antibodies that are normally absent. Genetic factors may also play a role in the predisposition to rheumatoid factor production.

The biologic function of rheumatoid factor is not well defined. It is known, however, that complexes of rheumatoid factor activate complement and thereby can induce an inflammatory response. This may occur in synovium, synovial fluid, or elsewhere in the body.

The specific sites of rheumatoid factor production in nonrheumatoid disease have not been investigated but presumably are similar to other sites of antibody production. In rheumatoid arthritis, however, the predominant source of rheumatoid factor is synovium with lesser amounts produced by bone marrow, lymph nodes, subcutaneous nodules, and spleen.

The rheumatoid factors produced by patients with rheumatoid arthritis are usually present in higher titer, are more heterogeneous, and react better with animal IgG than rheumatoid factors produced in patients with other diseases. The Rose–Waaler method using sheep cell agglutination is thus more specific for rheumatoid arthritis, although the titers are generally much lower than those obtained with the latex or bentonite methods.

Clinical Significance

Rheumatoid factor production has been shown in a variety of diseases other than rheumatoid arthritis. Most of these diseases are associated with chronic antigenic stimulation or polyclonal B cell activation. Patients with connective tissue diseases such as systemic lupus erythematosus, mixed connective tissue disease, scleroderma, and Sjögren's syndrome frequently have elevated serum rheumatoid factor titers. Rheumatoid factor is often elevated in noninfectious hyperglobulinemic conditions such as hypergammaglobulinemic purpura, cryoglobulinemia, chronic liver disease, sarcoidosis, and idiopathic pulmonary fibrosis. Many infectious diseases also result in rheumatoid factor elevation. Examples include acute viral infections such as mononucleosis or hepatitis; chronic bacterial diseases such as tuberculosis, leprosy, syphilis, and subacute bacterial endocarditis; and parasitic infections.

The presence of rheumatoid factor is thus not diagnostic for any specific disease. Mild elevations of rheumatoid factor are a normal result of aging. In general, high titers of rheumatoid factor are more frequently found in patients with rheumatoid arthritis than in patients with other rheumatic and nonrheumatic diseases.

Most patients with rheumatoid arthritis have elevated rheumatoid factor titers within a year of onset of the disease, although approximately 10 to 20% remain rheumatoid factor negative. The diagnosis of rheumatoid arthritis is made on the basis of signs and symptoms of chronic inflammatory arthritis rather than by the presence of rheumatoid factor. Nevertheless, high titers of rheumatoid factor in rheumatoid arthritis patients are associated with more severely destructive arthritis and with extraarticular manifestations of the disease including subcutaneous nodules, sicca symptoms, Felty's syndrome, and pulmonary or cardiac complications. Elevated titers of IgG rheumatoid factor are frequently found in patients with rheumatoid vasculitis.

Treatment of rheumatoid arthritis with disease-modifying agents may cause reduced rheumatoid factor production; however, nonsteroidal anti-inflammatory agents such as aspirin usually do not have any effect on rheumatoid factor levels. The activity of rheumatoid arthritis should be monitored by physical findings, roentgenographic changes, and other laboratory studies rather than by changes in rheumatoid factor titer. Previous studies have shown, however, that successful treatment of infectious diseases such as subacute bacterial endocarditis does result in reduction of rheumatoid factor production.

References

Carson DA. Rheumatoid factor. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;198–207.

Egeland T, Munthe E. Rheumatoid factors. Clin Rheum Dis 1983;9:135–60.

Johnson PM, Faulk WP. Rheumatoid factor: its nature, specificity, and production in rheumatoid arthritis. Clin Immunol Immunopathol 1976;6:414–30.

Masi AT, Maldonado-Cocco JA, Kaplan SB, et al. Prospective study of the early course of rheumatoid arthritis in young adults. Semin Arthritis Rheum 1976;5:299–326.

Antinuclear Antibodies

Definition

Antibodies that bind to nuclear and cytoplasmic components of mammalian cells are present in the sera of patients with many of the rheumatic diseases. Extensive study has elucidated the antigenic determinants of many of these antibodies and provided correlation of specific antigen–antibody systems with specific diseases. The antinuclear antibodies (ANA) most frequently detected in the serum of rheumatic disease patients are listed in Table 167.3. The antigens to which the antibodies react are named by several methods. Some are cell constituents, and some are named after the disease with which they are associated. Others, such as Sm, Ro, La, Ha, and Jo, are named by the first two letters of the name of the patient in whom the monospecific antibody was first identified. Several methods are available for the detection or quantification of these autoantibodies. Depending on the type and sensitivity of the test used, the results are reported as "positive" or by other quantitative or qualitative means.

Technique

The test first reported to detect antinuclear antibodies, the lupus erythematosus (LE) cell test, was developed by Hargraves and colleagues. It has subsequently been replaced by more sensitive and specific tests. It is rapid and can be done in any laboratory, but it is difficult to perform, and the results are difficult to interpret by persons who are not experienced. Whole blood, serum, or other biologic fluid (e.g., pleural, pericardial, or synovial fluid) can be used. The blood is incubated with a source of traumatized substrate cells and a source of viable phagocytic cells, usually polymorphonuclear white blood cells (PMNs). If antinuclear antibody to nucleoprotein is present in the test serum, it coats the nonviable nuclei and facilitates ingestion of the nucleus by the viable phagocytic cells. The result is a bluish homogeneous body in a PMN with the nucleus of the PMN crowded peripherally.

The indirect immunofluorescent test (IFA) is currently the method of choice to screen for the presence of antinuclear antibodies. Commercial kits containing tissue sections or cell culture monolayers are available for this test. A section of mammalian tissue or a layer of cells from a human tissue culture line is applied to a slide. The serum to be tested is flooded over the cells, and antibodies with specificity for nuclear or cytoplasmic antigens bind to those antigens. The serum is washed from the slide, but the antinuclear antibodies remain bound. Next the slide is treated with a solution containing fluorescein-tagged anti-human Ig that binds to the antibodies from the patient serum that bound the nuclear/cytoplasmic antigens. The slide is again washed and is then ready for viewing under a fluorescent

Table 167.3
Antinuclear Antigen–Antibody Systems in Rheumatic Disease

Cellular antigen	Disease associated with antibodies to the antigen[a]	Method used for detection of antibodies to the antigen	Nuclear immunofluorescent pattern of autoantibodies[b]
DNA-histone complex (nucleoprotein)	SLE, RA, CAH	LE cell phenomenon, IFA	Homogeneous/peripheral
DNA			
Double-stranded	SLE	IFA, RIA, *Crithidia* IFA, ELISA, CIEP, hemagglutination	Peripheral
Single-stranded	SLE, drug-induced SLE, CAH, infectious mononucleosis, RA	IFA, RIA, ELISA, hemagglutination	Homogeneous
Histones	SLE, drug-induced SLE	Histone-reconstituted IFA, ELISA	Homogeneous
Nuclear ribonucleoprotein (nRNP)	SLE, MCTD	IFA, hemagglutination, immunodiffusion[c], CIEP	Speckled
Sm	SLE	IFA, immunodiffusion, CIEP	Speckled
SS-A, Ro	SLE, Sjögren's syndrome, RA	IFA on human tissue culture cell lines, immunodiffusion, CIEP	Speckled (cytoplasm and nucleus)
SS-B, La, Ha	SLE, Sjögren's syndrome, RA	IFA, immunodiffusion, CIEP	Speckled
Scl-70	Scleroderma	IFA, immunodiffusion	Speckled
Centromere	CREST variant of scleroderma, scleroderma	IFA on human tissue culture cell lines	Centromere
Nucleolar (RNA)	Scleroderma	IFA	Nucleolar
PM-1, PM-Scl	Polymyositis-scleroderma overlap	IFA, immunodiffusion	? Nucleolar, speckled
Jo-1, Jo-2	Polymyositis	IFA, immunodiffusion	? Speckled
RANA	RA	Immunodiffusion, IFA on human tissue culture cell lines	Speckled (nucleus and cytoplasm)

[a]Most common disease associations.
[b]Cytoplasmic fluorescent patterns are indicated where appropriate.
[c]Ouchterlony double immunodiffusion.
DNA, deoxyribonucleic acid; RANA, rheumatoid arthritis nuclear antigen; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; CAH, chronic active hepatitis; MCTD, mixed connective tissue disease; CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias; RIA, radioimmunoassay; IFA, indirect immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; CIEP, counterimmunoelectrophoresis.

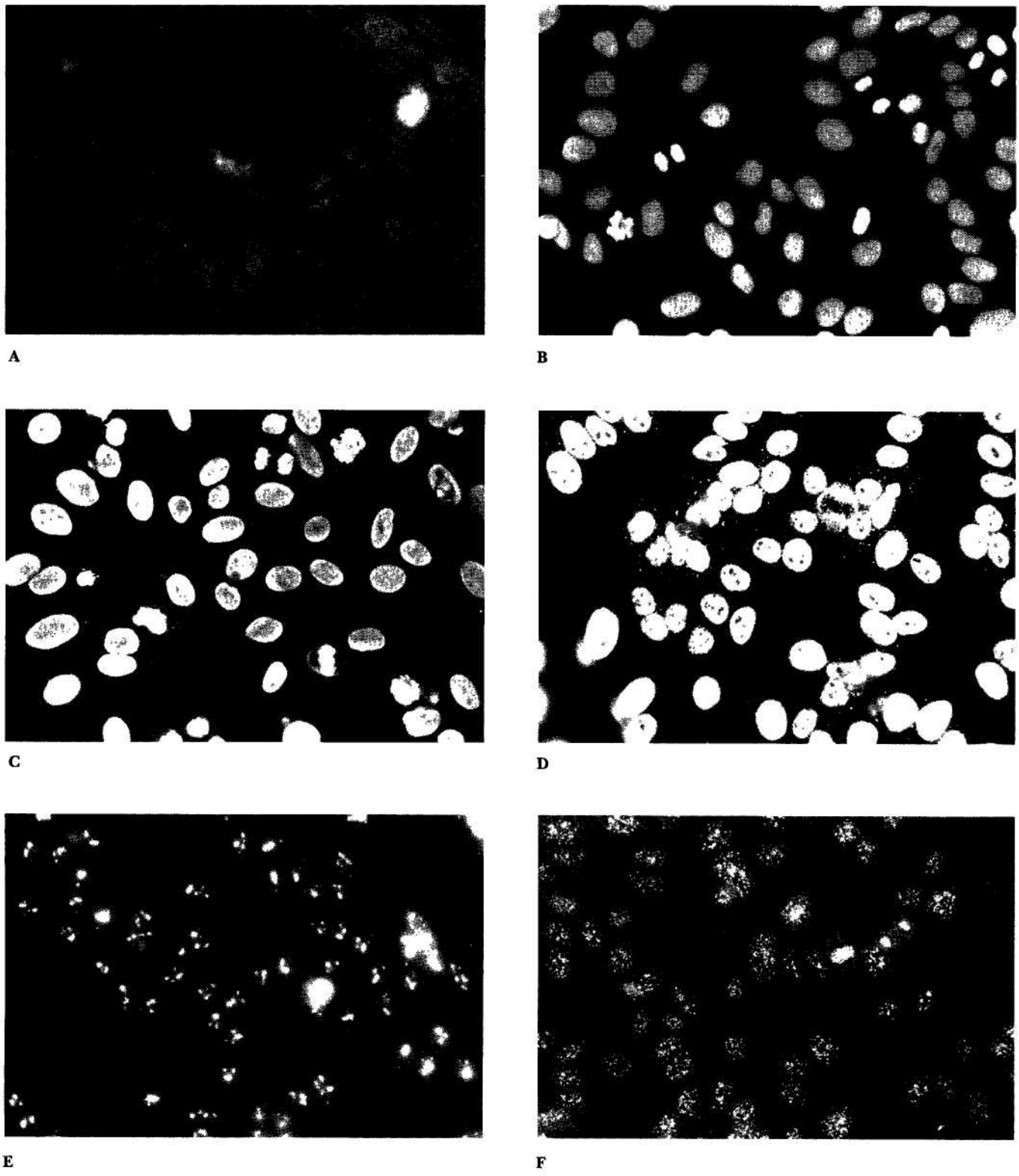

Figure 167.5
Immunofluorescent antinuclear antibody (IFA) test. (A) Negative control. (B) The homogeneous pattern associated with antibodies to nucleoprotein, including histones and single-stranded DNA. (C) Example of the peripheral or rim pattern associated with antibodies to double-stranded DNA. There is also homogeneous fluorescence, but the outer rim of most of the nuclei is more intensely fluorescent. (D) The speckled pattern associated with antibodies to many of the non-DNA nuclear antigens. (E) The nucleolar pattern. (F) Example of the fine nuclear speckled pattern associated with anticentromere antibodies. Cells from a human tissue culture line (Hep2) were used for these tests and are shown at high power (×400).

microscope. The test is first done at a screening serum dilution of 1:10 or higher. If fluorescence is seen, further dilutions are done, and the result given is the pattern of staining at the highest dilution of serum. Because of variations in substrates, laboratory conditions and techniques, and antigen–antibody systems determined, it is not possible to make a general statement about the significance of a certain titer or pattern. Often the pattern of fluorescence may vary with different dilutions of the same patient's serum. Examples of fluorescence patterns are shown in Figure 167.5, and general correlations of the patterns seen with certain antinuclear antibodies are listed in Table 167.3.

To detect serum antibodies to nuclear histones, the IFA test for antihistone antibodies requires additional procedures. Three slides containing tissue sections such as mouse kidney are used. One is prepared as described above, and the other two are initially treated with an acid solution to extract histones. One of the acid-treated sections is reconstituted with histones, and both slides are then prepared as described above. If antibodies to histones are present in the patient's serum, a fluorescent pattern should be seen on the routine IFA slide and the histone-reconstituted slide but not on the acid-extracted slide.

Another IFA test has been developed that is sensitive and specific for antibodies to double-stranded DNA. The hemoflagellate *Crithidia luciliae,* which has a kinetoplast containing only circular double-stranded DNA, is used as the substrate. The technique is similar to the IFA technique described above and can be enhanced to include characterization of the Ig classes in the human serum that bind the DNA or to determine the ability of the anti-DNA antibodies to fix complement.

The presence of antinuclear antibodies in the serum can be further identified by the use of immunodiffusion techniques. The Ouchterlony method of double immunodiffusion (Figure 167.6) is widely used for identification of specific antinuclear antibodies. A central well is cut into an agarose gel for the placement of a source of antigen such as calf thymus or other mammalian organ extracts. Other wells for the placement of serum from the patient and sera with known antibody specificity are made around the central well. The antigen and antibodies diffuse into the agarose, forming a precipitin line. If the antibody specificity of the patient's serum is the same as the reference serum, a line of identity is formed as a continuous precipitin line. Nonidentity is indicated by precipitin lines that cross each other. This method is relatively insensitive and is not quantitative but is useful in identification of many antinuclear antibodies for which other methods are not available. It cannot be used for detection of anti-DNA antibodies because DNA does not diffuse into agarose.

Antibodies to DNA and other acidic antigens such as Sm, RNP, SS-A, and SS-B can be detected by counterimmunoelectrophoresis, which is a very sensitive test. For this method, antigen is placed in a cathodal (−) well, and dilutions of serum are placed in an anodal (+) well. With electrophoresis, the antigens and antibodies diffuse through the agarose to form one or more precipitin lines.

The passive hemagglutination technique can be used to detect antibodies to RNP or DNA. The appropriate antigen is coupled to the surface of sheep red cells and added to microtiter wells containing increasing dilutions of patient serum. The presence of antibody specific for the antigen causes visible agglutination of red cells, and the test result is expressed as the highest dilution at which hemagglutination is present.

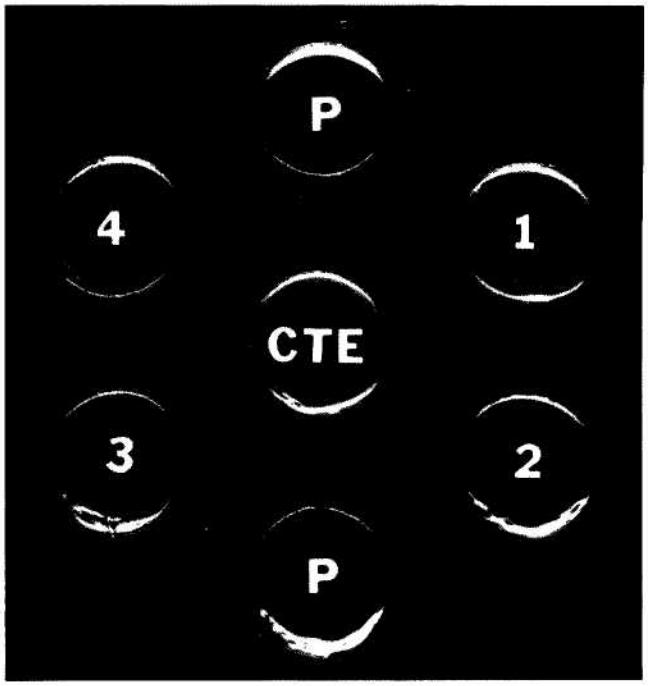

Figure 167.6
Double immunodiffusion by the Ouchterlony method. The center well containing calf thymus nuclear extract *(CTE)* is surrounded by wells into which prototype serum with specificity for RNP *(P)* or patients' serum *(1–4)* has been placed. The precipitin lines represent antigen–antibody complexes. The line formed by serum 3 is identical with that of the prototype line (anti-RNP), but those formed by serum from patients 1 and 4 cross the adjacent lines and are nonidentical, indicating other antigen–antibody reactions.

Radioimmunoassays (RIAs) are sensitive methods of quantifying specific antinuclear antibodies. For maximal specificity, the nuclear antigen must be purified, so this method is not available clinically for detection of many of the antinuclear antibodies. Antibodies to double- and single-stranded DNA can be detected by RIA. The most frequently used methods are the Farr assay and a modification of this assay using a nitrocellulose filter. For the Farr assay, the test serum is incubated with radiolabeled double- or single-stranded DNA in a saturated ammonium sulfate solution. Insoluble antigen–antibody complexes are precipitated if anti-DNA antibodies are present in the serum, which can then be separated from the supernatant by centrifugation. The amount of radioactive DNA bound by antibody in the precipitate is compared to that remaining unbound in the supernatant, and the results are expressed as percentage of DNA-binding activity. The nitrocellulose technique is a modification of the Farr assay in which the antigen–antibody complexes are separated from the supernatant by filtration. The radioactivity trapped on the filter in the form of the DNA complexes is compared to that in the unbound form in the supernatant. The results are expressed as nanograms of DNA bound per milliliter of serum. In other RIA methods the specific antigen may be coated onto plastic tubes or microtiter wells as discussed in the section on Rheumatoid Factor. The ELISA method (enzyme-linked immunosorbent assay) is highly sensitive and uses the same principles as the RIA methods. This method has the advantage of not requiring radioisotopes and is thus less expensive and safer. The detection of antinuclear antibodies

by ELISA requires the adsorption of nuclear (or cytoplasmic) antigens onto a solid medium, but is otherwise as described in the section on Rheumatoid Factor.

The "lupus band test" is a direct immunofluorescent test in which fluoresceinated antibodies to immunoglobulin, complement components, and other substances such as fibrinogen are placed on skin biopsy tissue. If immunoglobulin or complement has been deposited at the dermoepidermal junction, a band of fluorescence is observed.

Basic Science

The etiology of SLE and other autoimmune diseases associated with the production of antibodies with specificity for self-antigens remains to be determined. It is likely that many factors, including genetic predisposition superimposed with environmental influences, initiate the autoimmune process. Immunologic abnormalities reported in lupus patients include loss of suppressor T cell function and B cell hyperactivity when the disease is active. Whether some of the abnormalities have an etiologic role or occur as a result of the disease is not known.

SLE is the prototype autoimmune disease where the deposition of antigen–antibody complexes results in tissue damage. Anti-DNA antibodies are thought to have a role in the pathogenesis of SLE, and antibodies to SS-A antigen are probably directly responsible for congenital complete heart block in the fetuses of women whose serum contains these antibodies. A pathogenetic role for other antinuclear antibodies, such as those associated with mixed connective tissue disease, scleroderma, and polymyositis, has yet to be determined. The occurrence of these latter autoantibodies serves as a marker of the disease and allows characterization of subsets of patients in terms of genetic factors, clinical manifestations, and prognosis. Recent and ongoing studies of the molecular characterization and biological and biochemical function of many autoantigens such as RNP, Sm, SS-A, SS-B, and Scl-70 may elucidate the pathogenetic role of their autoantibodies in the future.

Clinical Significance

When the clinical presentation suggests that the patient may have a rheumatic disease associated with the production of antinuclear antibodies, the screening test of choice is the IFA for antinuclear antibodies. Most SLE patients have a positive test, many with serum dilutions greater than 1:100. If the screen is negative on organ tissue, it may be positive when repeated on tissue culture cells. The frequency of a positive test in normal individuals varies in different laboratories and increases with the age of the patient, so positive tests in the absence of clinical findings should not usually be pursued. When the IFA is positive, as in patients suspected to have SLE, drug-induced lupus, mixed connective tissue disease, or myositis, additional tests for specific antinuclear antibodies can be performed to allow a more specific diagnosis. For example, patients with drug-induced lupus who have positive IFA and antibodies to nuclear histones rarely have antibodies to double-stranded DNA.

In addition to being highly specific for the diagnosis of SLE, serum levels of antibodies to double-stranded DNA fluctuate with disease activity, especially of nephritis, and can be used to monitor treatment. The *Crithidia luciliae* IFA test and RIA tests are well suited for this use.

The "biologically false positive test for syphilis" (BFTS), which is known to occur in patients with SLE, results from antibody reactivity to the phospholipid cardiolipin. The occurrence of a positive test, confirmed by additional procedures to be negative for syphilis, is one of the classification criteria for SLE. A positive BFTS has recently been shown to be more significant in some patients than was previously appreciated. Antibodies to cardiolipin and other phospholipids can also be determined by ELISA. The "lupus anticoagulant," another antiphospholipid antibody, the BFTS, and anticardiolipin antibodies are associated with arterial and venous thrombosis, recurrent fetal losses, thrombocytopenia, and possibly nervous system abnormalities in some patients.

The "lupus band test" is positive in most SLE patients if skin is biopsied from lupus skin lesions or sun-exposed skin. The most significant positive result is the presence of immunoglobulin deposition at the dermoepidermal junction of non-sun-exposed, non-lesional skin from a patient with suspected SLE. This finding appears to correlate with the presence of severe disease including nephritis. The test may also be positive in some patients with mixed connective tissue disease.

The diagnosis of mixed connective tissue disease is made in patients with overlapping features of SLE, rheumatoid arthritis, polymyositis, and scleroderma. The titer of antibodies to RNP is usually very high and may or may not fluctuate with disease activity.

In patients with scleroderma, the presence of anticentromere antibody identifies patients with the CREST variant of scleroderma (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias). While these patients have been shown to develop disease more slowly than those with the more rapidly progressive form, they are at risk of developing pulmonary and other systemic manifestations, but have a lower incidence of renal disease. The presence of anticentromere antibodies in patients presenting with isolated Raynaud's phenomenon may indicate the future development of the CREST syndrome.

Many patients with rheumatoid arthritis have positive IFA on tissue substrates, usually with speckled, homogeneous, or even peripheral patterns. These patients usually have positive tests for rheumatoid factor and severe disease. Previous studies have shown that rheumatoid factors may cross react with nuclear antigens.

Recent studies have identified IgG antibodies to cytoplasmic components of neutrophils in patients with Wegener's granulomatosis and other systemic vasculitides. A negative anticytoplasmic antibody (ACPA) titer does not rule out the presence of these diseases, but when it is positive, it may be useful to monitor disease activity, since the titer may change with remissions and exacerbations.

References

Alarcon-Segovia D. Antibodies to nuclear and other intracellular antigens in the connective tissue diseases. Clin Rheum Dis 1983;9:161–76.

Alspaugh M, Tan EM. Serum antibody in rheumatoid arthritis reactive with a cell-associated antigen. Arthritis Rheum 1976; 19:711–19.

Arnett FC, Hirsch TJ, Nishikai M, et al. The Jo_1 antibody system.

Clinical and immunogenetic associations in myositis. J Rheumatol 1981;8:925–30.
Barada FA Jr, Andrews BS, Davis JS IV, et al. Antibodies to Sm in patients with systemic lupus erythematosus. Arthritis Rheum 1981;24:1236–44.
Chubick A, Sontheimer RD, Gilliam JN, et al. An appraisal of tests for native DNA antibodies in connective tissue diseases: clinical usefulness of the *Crithidia luciliae* assay. Ann Intern Med 1978;89:186–92.
Davis JS IV. Antinuclear antibodies (ANA). In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. Philadelphia: W.B. Saunders 1981;691–709.
Fritzler MJ, Tan EM. Antibodies to histones in drug-induced and idiopathic lupus erythematosus. J Clin Invest 1978;62:560–67.
Hargraves MM, Richmond H, Morton R. Presentation of two bone marrow elements: "tart" cell and "L.E." cell. Proc Staff Meet Mayo Clin 1948;23:25–28.
Harris EN, Hughes RV. Antiphospholipid antibodies. In: McCarty DJ, ed. Arthritis and allied conditions. 11th ed. Philadelphia: Lea and Febiger, 1989;1068–1079.
Hughes GRV, Harris EN, Gharavi AE. The anticardiolipin syndrome. J Rheumatol 1986;13:486–89.
Litsey SE, Noonan JA, O'Connor WN, et al. Maternal connective tissue disease and congenital heart block. N Engl J Med 1985;312:98–100.
McCarty GA, Valencia DW, Fritzler MJ. Antinuclear antibodies: Contemporary techniques and clinical application to connective tissue diseases. New York: Oxford University Press, 1984.
Notman DD, Kurata N, Tan EM. Profiles of antinuclear antibodies in systemic rheumatic diseases. Ann Intern Med 1975;83:464–69.
Pisetsky DS. Mechanisms of antinuclear antibody production in the rheumatic diseases. Clin Rheum Dis 1987;13:569–581.
Reichlin M. Antinuclear antibodies. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;208–25.
Schur PH. Clinical features of SLE. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;1101–29.
Sharp GC, Irvin WS, Tan EM, et al. Mixed connective tissue disease—an apparently distinct rheumatic disease syndrome associated with a specific antibody to an extractable nuclear antigen (ENA). Am J Med 1972;52:148–59.
Sharp GC, Irvin WS, May CM, et al. Association of antibodies to ribonucleoprotein and Sm antigens with mixed connective-tissue disease, systemic lupus erythematosus and other rheumatic diseases. N Engl J Med 1976;295:1149–54.
Specks U, Wheatley CL, McDonald TJ, Rohrbach MS, DeRemee RA. Anticytoplasmic autoantibodies in the diagnosis and follow-up of Wegener's granulomatosis. Mayo Clin Proc 1989;64:28–36.
Tan EM, Rodnan GP, Garcia I, et al. Diversity of antinuclear antibodies in scleroderma: anticentromere antibody and its relationship to CREST. Arthritis Rheum 1980;23:617–25.
Woods VL Jr, Zvaifler NJ. Pathogenesis of systemic lupus erythematosus. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;1077–1100.

The Complement System

Definition

The complement system is composed of at least 18 different proteins, most of which are alpha- and beta-globulins. These interact to promote or inhibit a cascade of enzymatic reactions resulting in cell lysis, initiation of the inflammatory response, and other phenomena. Two initial pathways of activation (the classic pathway and the alternative pathway) result in activation of the terminal sequence (Figure 167.7). The components of the classic pathway are identified by numbers preceded by *C:* C1, C4, C2, C3, C5, C6, C7, C8, C9. The alternative pathway components are indicated by capital letters, such as factor B, factor D, and factor P (properdin). As the components are acted on by previous components or other molecules, enzymes are formed. These enzymes are indicated by a bar over the protein complex components (e.g., $C\overline{5678}$, $\overline{C3b}$, $\overline{B}$). Cleavage products of enzymatic activity are indicated by lowercase letters (e.g., C3b, C5a). Inactive products are indicated with an "*i*," (e.g., C3bi).

The ranges of normal serum levels of most of the complement proteins are known and are listed in many of the references at the end of this section. Serum levels of C3 are the highest, followed by C4. The method used to determine complement component levels and the normal ranges for individual laboratories are variable. The most commonly used assay of total complement function is the total hemolytic assay (CH50). The results of this test are usually expressed in comparison with values obtained using normal sera, but the absolute results may differ greatly depending on performance conditions of the test.

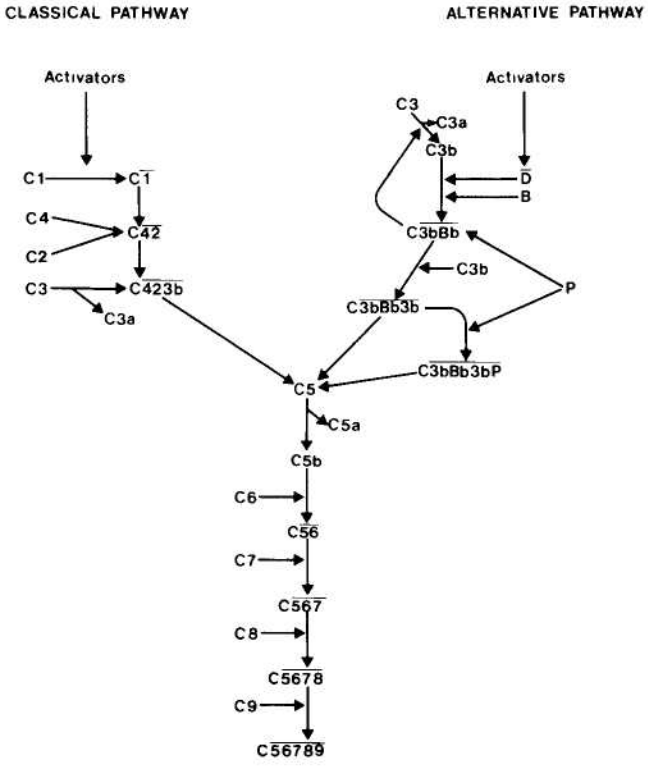

Figure 167.7
Simplified schematic of the classic and alternative pathways and the terminal sequence of complement activation.

Technique

Serum and other biologic fluid samples for complement determinations should be handled expeditiously to avoid decay of complement proteins. When serum is used for the detection of complement, the blood should be allowed to clot at room temperature, then be centrifuged and stored at −70°C until processed in order to preserve complement activity.

Serum complement protein concentrations can be assessed individually and quantitatively by radial immunodiffusion assays available commercially in kits. The most widely used assays are for C3 and C4, and these are sufficient in most clinical situations. The method for radial immunodiffusion is discussed in the section on C-reactive protein. For quantifying complement components, serum is placed in a well containing antibodies to a specific complement protein. The diameter of the precipitin ring formed by the complexing antigen (i.e., complement protein) and antibody is compared to predetermined or, preferably, simultaneously tested standards. This method is capable of detecting complement protein concentrations as low as 20 μg per milliliter of serum.

Some disadvantages of the immunodiffusion method should be recognized. As previously mentioned, there are no international standards, and wide variability in absolute results occur. Also, the cleavage products of complement proteins are measured by this assay and are not distinguished from active proteins.

The immunonephelometry and ELISA techniques have also been developed for quantification of some of the complement proteins, particularly C3 and C4.

The CH50 assay provides a means of screening for function of the total complement sequence by determining the ability of the patient's serum to lyse erythrocytes. Sheep red cells are coated with rabbit anti-sheep erythrocyte antibodies; usually 1×10^8 red cells are used. A CH50 unit is arbitrarily defined as the amount of complement required to lyse 50% of the red cells. The number of red cells lysed is determined by spectrophotometric absorbance of released hemoglobin, which has a linear relationship to complement protein levels in the 50% lysis range. The amount of the patient's serum required to lyse 50% of the red cells is determined. This is correlated with the amount of complement known to be required for 50% lysis. Thus, larger amounts of patient serum are required to achieve lysis when the complement activity in the serum is reduced.

This test is influenced by many variables and is not precisely quantitative. It is especially useful, however, in screening for homozygous classic pathway complement deficiencies, since lysis will not occur if one component is absent. Patients with heterozygous (incomplete) deficiencies may have a normal CH50. To test for alternative pathway deficiency, lysis of rabbit erythrocytes that are not coated with antibody may be used.

When hereditary deficiencies of complement proteins are suspected from the clinical picture and the results of the CH50 assay, additional tests for other classic complement proteins are appropriate and can be determined by the immunodiffusion or other methods. Levels of $C\overline{1}$ inhibitor can also be determined by this method. The CH50 assay may also be used to assess the function of specific complement components, but the test is technically difficult.

Other assays have been developed recently to measure the events of complement activation more precisely. These tests can measure the limited proteolytic cleavage events of both pathways, the activation-related changes in the properties of the complement pathway components, and the quantity of specific protein complexes that are generated by certain stages of complement activation. These tests are still used primarily for research, but, since they can be used with all body fluids, they may be very applicable for future clinical use.

Basic Science

The classic complement pathway can be activated by antigen–antibody complexes, by Ig–Ig complexes, or by substances such as DNA, staphylococcal protein A, CRP, and some enzymes. Of the immunoglobulins, IgM is the most efficient activator of the classic pathway, followed by IgG_3, IgG_1, and IgG_2; IgG_4, IgA, IgD, and IgE do not activate the classic sequence. The immunoglobulin or other substances bind directly to C1, which consists of three protein molecules—C1q, C1r, and C1s. C1q has the binding site for the Fc portion of Ig. This binding is followed by the activation of C1s by $C\overline{1}r$; C1s acquires proteolytic enzyme activity that activates C2 and C4 to form $C\overline{42}$. When C4 is cleaved into C4a and C4b, C4b can attach to cells or membranes briefly. $C\overline{42}$, also called C3 convertase, cleaves and activates C3. C3 is cleaved into C3a and C3b, the latter having the ability to bind to membranes. The attachment of C3b near $C\overline{42}$ leads to the formation of $C\overline{423}$, which has the ability to cleave C5, the initial component of the terminal sequence.

The alternative complement pathway is activated by IgA, some IgG and IgE, other substances such as lipopolysaccharides, plant and bacterial polysaccharides, and some enzymes. The biologic advantage of this pathway is that it is activated by microorganisms in the absence of antibody. The reactions in this pathway are analogous to those in the classic pathway but involve different proteins. Factor D is a protease that is present in plasma in its active form and functions similarly to $C\overline{1}s$. $\overline{D}$ acts on C3 and factor B to form the alternative pathway C3 convertase, $C\overline{3b}\overline{Bb}$, which is stabilized by factor P (properdin). $C\overline{3b}\overline{Bb}$ is unstable, but decay leads to regeneration of C3b that is capable of interacting again with B and D to generate new C3 convertase. C3 convertase cleaves C3 to form $C\overline{3b}\overline{Bb}\overline{3b}$, which, similar to $C\overline{423}\overline{b}$ in the classic pathway, acts as C5 convertase. From this point the terminal sequences of both pathways are the same.

The cleavage of C5 by C5 convertases results in C5a and C5b. C5b combines with membranes and then with C6 to form $C\overline{56}$, which reacts with C7. The $C\overline{5b67}$ complex is the first stage of the membrane attack complex. When this complex reacts with C8, $C\overline{5b678}$ can cause membrane damage. The addition of C9 to form $C\overline{56789}$ results in a stable complex that causes cell lysis when bound to the surface of cells.

Control proteins exist for both the classic and alternative pathways. These act to prevent continual complement activation. In the classic pathway, C1 esterase inhibitor ($C\overline{1}$ INH) binds to C1s or C1r and can prevent C4 and C2 cleavage. $C\overline{1}$ INH also inhibits the activity of kallikrein, factor XI, plasmin, and activated Hageman factor, which can otherwise activate C3 to form C3b. A C4 binding protein (C4-BP) binds to C4b and enables another inhibitor (C3b inactivator) to bind; this results in inability of C4 to form $C\overline{42}$. The control proteins for the alternative pathway are factor I (which leads to inactivation of C3b) and factor H (which can bind 3b and render it more susceptible to cleavage by I and also compete with binding by factor B).

Another protein that affects the activity of the alternative pathway is C3 nephritic factor (C3NeF). C3NeF is an autoantibody that binds the alternative pathway C3 convertase, $C\overline{3bBb}$. Its function is similar to that of properdin, which is to stabilize C3 convertase and increase the efficiency of the pathway proteins.

In addition to the end result of the complement pathways (i.e., cell lysis), many of the cleavage products of complement components have biologic functions, which are listed in Table 167.4.

The importance of C3b as a biologically active fragment should be emphasized. Many different cell types possess C3b receptors. These include B lymphocytes, erythrocytes, platelets, and phagocytic cells such as polymorphonuclear leukocytes, monocytes, and macrophages. When immune complexes or microorganisms are coated with C3b in the presence of IgG that can bind to Fc receptors, phagocytosis by C3b receptor-bearing cells is enhanced. In the absence of IgG, C3b promotes adherence. The complement system thus provides a mechanism for initiating the inflammatory response, for cell lysis, and for removal of microorganisms and immune complexes.

Clinical Significance

The importance of the complement system is most apparent in patients who are congenitally deficient in complement components or regulators of the complement pathways. Patients with hereditary deficiency of $C\overline{1}$ INH, C1, C2, C4, C5, and C8 can manifest systemic lupus erythematosus or lupus-like illness. Raynaud's phenomenon has been reported to occur with deficiency of C6 and C7. Deficiencies of C2, C3, or C5–9 are associated with recurrent infections. Patients with deficiency of C3 have recurrent pyogenic infections. C5 dysfunction is associated with Leiner's disease (gram-negative infections and eczema in children), and deficiency of any of the terminal complement components C5–9 is associated with increased susceptibility to Neisserial infections including gonococcal and meningococcal illnesses. C2 deficiency is the most frequent of the complement component deficiencies.

Deficiency of $C\overline{1}$ INH (C1 esterase inhibitor) is associated with hereditary angioneurotic edema, characterized by recurrent subepithelial swelling of the skin and mucous membranes of the gastrointestinal and upper respiratory tracts. Attacks are often precipitated by local trauma. Serum levels of C4 and C2 are usually depressed in these patients, especially during attacks, due to the constant activation of C1 and subsequent utilization of its substrates C4 and C2.

Some of the complement components, especially C3, are elevated as acute phase reactants during inflammatory disease. In such situations, levels of these components will be elevated unless increased complement utilization is occurring.

The diseases most frequently associated with altered complement levels due to increased activation of the complement system are chronic inflammatory disorders in which immune complexes play an important pathogenetic role. SLE is the most common rheumatic disease associated with low serum complement levels, especially C3, C4, and C1q, with resulting reduction in CH50. Complement levels are usually lower in patients with active SLE, especially those with nephritis. The monitoring of serial complement levels thus provides a useful parameter of disease activity in most patients. Often complement levels may fall prior to a flare of disease activity, and patients who appear well but have falling complement levels should be followed more frequently for signs of disease activity. Similarly, serum complement levels (C3, C4, or CH50) are useful in monitoring the response to treatment.

Other rheumatic diseases in which serum complement levels may be reduced include vasculitis associated with rheumatoid arthritis and Sjögren's syndrome; essential mixed cryoglobulinemia; and necrotizing vasculitis, including some patients with polyarteritis nodosa. In some patients with vasculitis, including rheumatoid vasculitis, complement levels may normalize with clinical improvement. However, complement levels have been reported not to correlate with disease activity in essential mixed cryoglobulinemia.

Nonrheumatic diseases may also cause hypocomplementemia. Decreased serum complement levels may be found in patients with poststreptococcal glomerulonephritis, membranoproliferative glomerulonephritis associated with C3NeF, and idiopathic glomerulonephritis. Infectious diseases such as infective endocarditis, infected arteriovenous shunts, sepsis, some viremias, and some parasitemias can also cause increased complement consumption.

Several studies have evaluated the utility of measuring complement components in synovial, pleural, and pericardial fluids in the differential diagnosis of effusions. Complement levels in these fluids must be compared with measurements of total protein in the fluid in order to be valid. Furthermore, because measurements of specific complement components may detect inactive as well as functional components, the result may not give a valid indication of complement activity. CH50 determinations are more meaningful. Studies have shown that CH50 levels in synovial fluids of patients with inflammatory arthritis due to rheumatoid arthritis and SLE are lower than normal, whereas they are not decreased in degenerative joint disease or most other inflammatory arthritides. These tests are not recommended for the routine evaluation of synovial fluid, however, because they usually add little to the diagnostic

Table 167.4
Biologic Activities of Complement Components

Component	Biologic activity
C1	Virus neutralization
C4, C2 kinin	Increased vascular permeability
C4	Virus neutralization
C4b	Immune complex clearance
C4-binding activity	Immune complex lysis
C3	Virus neutralization
C3bBb	Chemotoxis of polymorphonuclear leukocytes
C3a	Histamine and serotonin release (anaphylatoxin)
C3b	Immune complex clearance, enhancement of phagocytosis
iC3b	Binding to glomerular, monocyte complement receptors
C3d	Lymphocyte complement receptor binding
C5a	Anaphylatoxin
C5b-9	Membrane damage, cell lysis

Sources: Adapted from George D, Glass D. Quantitation of complement proteins in rheumatic disease. Clin Rheum Dis 1983;9:177–98, and Ruddy S. Plasma protein effectors of inflammation: complement. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;9;241–52.

evaluation. CH50 and C4 levels have also been reported to be reduced in rheumatoid and SLE pleural effusions when compared with effusions associated with malignancy (see Hunder, 1972). The measurement of complement levels in cerebrospinal fluid is not practical or reliable, however, because of rapid decay of complement activity.

References

Cooper NR. The complement system. In: Stites DP, Stobo JD, Fudenberg HH, Wells JV, eds. Basic and clinical immunology. 6th ed. Norwalk and Los Altos: Appleton and Lange, 1987;114–27.

Frank HM. Complement in the pathology of human disease. N Engl J Med 1987;1525–30.

George D, Glass D. Quantitation of complement proteins in rheumatic disease. Clin Rheum Dis 1983;9:177–98.

Gorevic PD, Kassab HJ, Levo Y, et al. Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. Am J Med 1980;69:287–308.

Hunder GG, McDuffie FC, Hepper NG. Pleural fluid complement in systemic lupus erythematosus and rheumatoid arthritis. Ann Intern Med 1972;76:357–63.

Pekin JH Jr, Zvaifler NJ. Hemolytic complement in synovial fluid. J Clin Invest 1964;43:1372–82.

Petz LD, Sharp GC, Cooper NR, et al. Serum and cerebrospinal fluid complement and serum antoantibodies in systemic lupus erythematosus. Medicine 1971;50:259–75.

Ross SC, Densen P. Complement deficiency states and infection. Epidemiology, pathogenesis and consequences of neisserial and other infections in an immune deficiency. Medicine 1984;63:243–73.

Ruddy S. Complement. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;241–52.

Ruddy S. Complement deficiencies and the rheumatic diseases. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989; 1386–94.

Ruddy S, Gigli I, Austen KF. The complement system in man. N Engl J Med 1972;287:489–95,545–49,592–96,642–46.

Stites DP, Rodgers RPC. Clinical laboratory methods for detection of antigens and antibodies. In: Stites DP, Stobo JD, Wells JV, eds. Basic and clinical immunology. 6th ed. Norwalk and Los Altos: Appleton and Lange, 1987;241–84.

Immune Complexes

Definition

An immune complex is formed when an antibody combines with its antigen. In general, there are three major sites of immune complex formation. When antibody reacts with tissue cell antigens or antigens fixed in the intercellular space, the immune complex remains localized. Immune complexes may also be formed and deposited locally near the site of injected or secreted soluble antigen. When soluble antigens circulating in the intravascular space combine with antibody, circulating immune complexes are formed. These circulating immune complexes, depending on their physical and other pathogenetic properties, can be deposited in blood vessels or filtering membranes. In the normal individual, serum immune complex levels are very low.

Techniques

There are multiple tests for determining levels of serum immune complexes, and the absolute results may be variable among laboratories. The most frequently used methods available are antigen nonspecific and rely on the ability of immune complexes to interact with complement, immunoglobulin, or receptors for complement or immunoglobulin (Fc) on the surface of cells. Only the most widely used assays are described here.

The C1q binding assay measures the binding of C1q to immune complexes and may be performed as a solid-phase or fluid-phase assay. C1q interacts with complexed rather than monomeric IgG. Complexes containing IgM, IgG_1, and IgG_3 are bound well by C1q; those containing IgG_2 are bound moderately well; and those containing IgG_4 are not bound. In the solid-phase C1q binding assay, EDTA-treated serum is incubated in a plastic tube coated with C1q. The tube is then washed to remove the serum, leaving adhered complexes. Quantification of the complexes is achieved by adding radiolabeled anti-IgG, and the results are expressed as nanograms of IgG bound. The fluid-phase C1q binding assay is done by adding radiolabeled C1q to EDTA-treated serum in the presence of polyethylene glycol, which enhances precipitation of the C1q-bound immune complexes. After centrifugation, the radioactivity in the precipitate is determined, and the result is expressed as the percentage of C1q bound.

The conglutinin solid-phase binding assay relies on the ability of conglutinin, a bovine serum protein, to bind fixed C3. Immune complexes that contain C3 can be detected by this method. The test serum is incubated in plastic tubes coated with conglutinin. After washing, radiolabeled anti-Ig or radiolabeled staphylococcal protein A is added to the tube, and the amount of isotope bound to the immune complexes is measured.

Another solid-phase binding assay that detects immune complexes containing C3 is the anti-C3 assay. For this test (Fab)'2 fragments of anti-C3 antibodies are coated onto plastic tubes and incubated with serum. Quantification is done by using radiolabeled anti-Ig as in the C1q binding assay.

The monoclonal rheumatoid factor binding inhibition assay (MRF) is a solid-phase assay that uses monoclonal IgG rheumatoid factor coupled to microcrystalline cellulose. Aggregates of IgG are radiolabeled and incubated with the test serum on the cellulose. The amount of radiolabeled aggregated IgG bound to the cellulose is determined. If immune complexes are present in the test serum, they compete with the radiolabeled aggregates for binding, thus reducing the amount of radioactivity bound to the cellulose. This test is sensitive only for immune complexes containing IgG; if rheumatoid factor is present in the serum, it can interfere with the test.

Two assays in which the binding of immune complexes to cells is assessed are the Raji cell assay and the platelet aggregation assay. Raji cells are obtained from a human

lymphoblastoid cell line derived from Burkitt's lymphoma. They have high-affinity receptors for C3b, C3bi, C3d, and C1q, and low-affinity receptors for immunoglobulin Fc. This assay is useful for detecting immune complexes that fix complement. A dilution of the test serum is incubated with the cells to allow binding of complement-containing immune complexes. After washing the serum from the cells, the cell pellet is resuspended and treated with radiolabeled anti-IgG antibody. The cells are centrifuged and washed, and the radioactivity in the cell pellet is counted and compared to a standard curve. This test is very sensitive for IgG-containing immune complexes, but the presence of antinuclear or antilymphocyte antibodies can give false positive results.

The platelet aggregation assay is a very sensitive test based on the binding of immune complexes by Fc receptors on the surface of platelets. Freshly prepared human platelets are mixed with serial dilutions of test serum on microplates and incubated overnight at 5 to 9°C. The results are expressed as the highest dilution of serum that causes platelet aggregation when viewed with dark field illumination.

The technique for detecting mixed cryoglobulins, which are Ig-Ig immune complexes that precipitate in the cold, is discussed in the section on Cryoglobulins.

The detection of the antigen present in immune complexes is not done routinely as a screening test because of the wide variety of antigens possible. When the diagnosis is known, however, the presence of certain antigens such as hepatitis B can be determined.

To obtain the most accurate results and avoid alteration of the biologic activity or physical properties of immune complexes in processing blood specimens, several precautions should be taken. The specimen should be drawn into a clot tube without anticoagulant because heparin, EDTA, and citrate can interfere with some of the tests. After allowing the blood to clot at an ambient temperature of 25°C or greater, to avoid cryoprecipitation, the serum should be stored at −70°C if possible. Repeated freezing and thawing should be avoided.

Techniques are also available to determine the presence of immune complexes in tissue specimens. Fluorescein- or peroxidase-labeled antibodies to the immunoglobulin classes, complement components, fibrin, fibrinogen, or some antigens can be applied to tissue sections. Granular deposits of immunoglobulin, often in the presence of complement, are considered to represent immune complexes. Immune complexes may also be identified as electron-dense deposits by electron microscopy.

Basic Science

The formation of immune complexes is a normal result of the immune antibody response, and most immune complexes are not pathogenetic. The sources of antigens in immune complexes may be exogenous or endogenous. Exogenous sources include food, drugs, heterologous serum proteins, and microbial infection. Endogenous sources of antigens may be autoantigens, including autologous immunoglobulin, or tumor antigens.

The formation of immune complexes depends on the type of antigen, the affinity of antibody for its antigen, and the avidity or strength of the interaction of antibody with antigen. The ratio of antigen to antibody and other characteristics of the antigen and antibody determine the size and stability of the immune complex lattice. When antigen is in large excess, small soluble immune complexes are formed; these do not fix complement and do not initiate an inflammatory response. Immune complexes formed when excess antibody is present are more likely to be large and insoluble. Although these complexes can fix complement, they are removed from the circulation by phagocytic cells of the reticuloendothelial system before causing any harmful effects. The major site of immune complex clearance is in the liver by Kupffer cells. The immune complexes formed at or near equivalence, especially in slight antigen excess, have the greatest pathologic potential. These complexes are smaller and more soluble than those formed in antibody excess, and are more likely to persist in the circulation and be deposited in blood vessels or in filtering membranes such as the glomerular basement membrane. The amount of tissue damage and the chronicity of disease are determined by the degree of persistence of antigenic stimulation and subsequent immune complex formation and deposition.

The ability of immune complexes to activate complement results in many biologic phenomena, which are discussed in the section on Complement Systems. Activation of the alternative pathway results in solubilization of immune complexes, which facilitates their elimination. The binding of complement-fixed immune complexes to erythrocytes by their receptors for C3b/C4b(CR1) results in their removal by phagocytic cells that have C3b surface receptors. Immune complexes that are not fixed by complement and thus are not bound by CR1 on the erythrocyte are cleared by fixed tissue macrophages via Fc receptors but more slowly. The inflammatory response induced by complement activation also appears to enhance clearance of immune complexes in extravascular tissues.

The precise sequence of events that occurs in the pathologic process associated with immune complex deposition is not actually known. The activation of complement is apparently important, because serum complement levels are frequently decreased and complement components are often seen in immunofluorescent studies of tissues. The deposition of immune complexes in vessels is thought to occur following permeability changes of vessels caused by the release of vasoactive amines by platelets, basophils, and mast cells. The subsequent influx of polymorphonuclear leukocytes results in release of proteases, collagenases, elastases, and other tissue-damaging substances. Vascular damage and tissue destruction are the end result.

While antigens and antibodies that combine to form immune complexes may be different in the various diseases associated with immune complex formation, these diseases may have similar multisystem features. The common manifestations of circulating immune complex deposition include glomerulonephritis, vasculitis, arthritis, skin lesions such as purpuric rash and ulcers, pleuritis, and pericarditis. In contrast, local immune complex deposition is associated with tissue damage in isolated organs such as in autoimmune thyroiditis or antiglomerular basement membrane disease.

Clinical Significance

The demonstration of immune complexes and vascular damage in tissue such as skin or kidney may be helpful in establishing the diagnosis of diseases associated with immune complex deposition such as vasculitis. In contrast, single determinations of immune complex levels in the serum are of much less diagnostic value. The presence of high levels of immune complexes may aid in categorizing a dis-

ease, but is by no means diagnostic; and the results must be correlated with the clinical presentation. Serial immune complex levels may be of some value in monitoring fluctuations in disease activity or response to therapy, although other laboratory tests may be equally satisfactory and more readily available.

Immune complexes can be detected in the serum and/or tissues of patients with many of the rheumatic diseases and in a wide variety of infectious, malignant, and nonrheumatic chronic inflammatory conditions. The rheumatic diseases in which immune complexes are believed to have a pathogenetic role include SLE, adult and juvenile rheumatoid arthritis, mixed connective tissue disease, cutaneous vasculitis, mixed cryoglobulinemia, and possibly polyarteritis nodosa, Wegener's granulomatosis, and scleroderma.

Four of the immune complex assays are useful in discriminating between SLE and normal patients: the Raji cell assay (in the absence of lymphocytotoxic antibodies), the C1q liquid-phase binding assay, the platelet aggregation assay, and the C1q solid-phase assay. The last test has also been shown to correlate with disease activity, especially in renal disease. The antigenic component of the complexes is widely variable.

In rheumatoid arthritis, the presence of serum immune complexes is best detected by the Raji cell and C1q binding assays; they occur in up to 80% of patients. Because of the high prevalence of positive immune complex assays in rheumatoid arthritis, determination of immune complex levels is not generally thought to be helpful in making the diagnosis of rheumatoid vasculitis. Immune complexes are also detected in synovial fluid and are believed to have pathogenetic significance.

Increased levels of circulating immune complexes have been reported in nearly all patients with mixed connective tissue disease, 88% of them having a positive Raji cell assay and approximately 50% having a positive C1q binding assay or monoclonal rheumatoid factor inhibition assay. The antigen(s) in these complexes is unknown, and there is limited experience in defining the role of immune complexes and changes in serum levels with disease activity. The significance and precise pathogenetic correlation of circulating immune complexes in Wegener's granulomatosis are not yet known.

Patients with scleroderma have a multisystem autoimmune disease but generally do not have vasculitis. Positive results for circulating immune complexes have been reported using the Raji cell assay which correlated with the presence of serum antinuclear antibodies. Positive Clq binding assay tests have also been reported to be correlated with the presence of serum rheumatoid factor and pulmonary disease.

Hepatitis B surface antigen has been identified in the vascular lesions of polyarteritis nodosa, and patients with this disease have been reported to have circulating immune complexes.

References

Dixon FJ, Cochrane CG, Theofilopoulos AN. Immune complex injury. In: Samter M, Talmadge DW, Frank MM, Austen KF, Claman HN, eds. Immunological diseases. 4th ed. Boston: Little, Brown, 1988;233–259.

Doyer E, Lambert PH. Immune complexes. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 2nd ed. Philadelphia: W.B. Saunders, 1985;680–90.

Holla JT, Volanakis JE, Schrohenloher RE. Circulating immune complexes in mixed connective tissue disease. Arthritis Rheum 1979;22:484–89.

Kimberly RP. Immune complexes in the rheumatic diseases. Rheum Dis Clin N Am 1987;13:583–96.

Lakha S. Immune complexes. Clin Rheum Dis 1983;9:199–226.

Lambert PM, Dixon FJ, Zubler RH. A WHO collaborative study for the evaluation of 18 methods for detecting immune complexes in serum. J Clin Lab Immunol 1978;1:1–15.

Levinsky RJ. Methods of detecting circulating immune complexes. In: Lachman PJ and Peters DK, eds. Clinical aspects of immunology, 4th ed. Oxford: Blackwell Scientific Publications 1982;398–413.

Lloyd W, Schur PH. Immune complexes, complement and DNA in exacerbations of systemic lupus erythematosus (SLE). Medicine 1981;60:208–17.

Nydegger UE, Lambert PH. The role of immune complexes in the pathogenesis of necrotizing vasculitides. Clin Rheum Dis 1980;6:255–78.

Theofilopoulos AN. Immune complexes. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology, 3rd ed. Philadelphia: W.B. Saunders, 1989;226–39.

The Major Histocompatibility Complex and Human Leukocyte Antigens

Definition

The major histocompatibility complex (MHC) is located on the short arm of chromosome 6 in humans. The genes coded in this region determine the structure of antigens found on the cell surface of human leukocytes (HLA) and other cells. There are three major classes of HLA: Class I, which includes gene products encoded in the A, B, and C loci; Class II, which includes molecules encoded in the D region genes; and Class III, which includes some complement molecules. There are three subregions of the D locus, which are called DP (formerly SB), DQ, and DR. The DQ or DR regions have been shown to code for the D-region alloantigen systems MB, MT, DC, Te, and BR. Products of the A, B, and C loci are glycoprotein antigens associated with β_2-microglobulin and are on the surface of all nucleated cells and platelets. Products of the D region loci are composed of two polypeptide chains (α and β) found on the surface of B cells, macrophages/monocytes, activated T cells, and some other cell types. Multiple alleles of many of the gene loci have been identified. The group of alleles located on a single chromosome of either maternal or paternal origin is called a *haplotype*. If the alleles are the same, the individual is homozygous for that gene; if the alleles are different, heterozygous. The genes themselves define the genotype of an individual, and the expression of the genotype is the phenotype. Usually both alleles are expressed and are thus codominant. The World Health Organization HLA Nomenclature Committee is responsible for officially approving each allele. Each antigen is named according to

the gene locus by a letter, and the allele by a number (e.g., B27, DR4). The letter *w* after the locus designation indicates that the antigen has been recognized only provisionally by the nomenclature committee, except in the case of the C loci where *w* is used to distinguish HLA antigens from the terminology used for complement components.

Technique

The technique for identifying Class I antigen specificities requires the use of alloantisera with antibody activity against a specific HLA. The sera are usually obtained from multiparous women who have been exposed to a foreign HLA. The standard technique is a microcytotoxicity assay. Multiple alloantisera with specificity for HLA-A, -B, and -C antigens are placed in wells of microtiter plates and incubated with peripheral blood lymphocytes from an individual. Complement is added, and following further incubation, a dye is added that is taken up by dead cells killed by the alloantibodies. Only the alloantisera with specificity for an antigen on the lymphocyte surface can elicit killing of cells. The HLA phenotype can thus be determined by the pattern of reactivity as determined by cell killing.

Similarities or differences in Class II antigens are determined by the one-way mixed lymphocyte reaction (MLR). In this assay lymphocytes from one individual are cultured with lymphocytes from another. The stimulator lymphocytes are treated with irradiation or Mitomycin C so that they do not respond. Differences in haplotypes are then determined by lymphocyte proliferation and uptake of tritiated thymidine, which is added to the culture. This method is adequate for determining histocompatibility for transplantation, but not for genetic analysis, since a proliferative response occurs between heterozygous lymphocytes that differ by only one allele. For genetic analysis, homozygous stimulator cells must be used.

HLA-DR and DQ antigens on the surface of B lymphocytes can be defined by B cell alloantisera. The technique is similar to that used for Class I antigens except that enriched B cells are used. Monoclonal antibodies with activity against many of the Class I and Class II antigens have been developed and can be used for identification of a specific antigen instead of alloantisera. HLA-DP antigens (DPw1-DPwb) can be identified and typed by primed lymphocyte typing. The primed lymphocytes are prepared by incubation with stimulator cells which are matched for HLA-A, -B, -C, -D, -DR, and -DQ antigens and differ only at DP. Once these cells are primed, they can be cultured with unknown cells; the uptake of tritiated thymidine by the primed cells indicates that the unknown cells have the DP antigen to which the primed lymphocytes were sensitized.

Basic Science

The functions of Class I and Class II antigens are known largely because of studies in animals; they appear to be similar in humans. Generally, the Class I antigens function as recognition antigens for graft rejection by cell-mediated lysis by killer T lymphocytes. Perhaps more important, the Class I antigens function as recognition sites for viruses. In order for cytotoxic T lymphocytes to kill virus-infected cells, they recognize the antigen in the context of Class I antigen, and the cytotoxic T lymphocytes must bear the identical Class I antigen. Class II antigens function in the presentation of antigen to T cells by macrophages and as recognition sites for interaction between immunocompetent T and B cells.

The reason(s) for the association of certain HLA antigens with specific diseases is unknown. One hypothesis is that the presence of specific cell surface antigens determines the type of response or lack of response to a foreign (or self) antigen, thereby leading to a pathologic condition such as the production of autoantibodies. Another hypothesis is that specific HLA antigens act as receptors for agents such as viruses, toxins, or other foreign substances that are etiologic for certain diseases. A third possibility is that some HLA antigens are structurally and immunologically similar to an agent that can cause disease. If the agent is recognized as "self" and no immune response results, the agent could then cause its pathologic effects without interference. An alternative response could be the recognition of the exogenous agent as foreign, thereby resulting in an immune response that cross reacts with "self," resulting in autoimmune phenomena.

Another mechanism by which HLA genes may predispose to autoimmunity is called *transcomplementation* or *transassociation*. If certain α and β chains from different Class II molecules become paired, an immune response may result that is more likely to cause disease. This has been shown in Sjögren's syndrome/SLE in which the highest autoantibody levels are found in DQw1/DQw2 heterozygotes.

Clinical Significance

Some of the rheumatic diseases known to be associated with certain HLA antigens are listed in Table 167.5. The determination of HLA types in patients with diseases of unknown etiology is done primarily for research purposes in attempts to characterize a disease better and understand its etiology.

The most closely linked association is that of HLA-B27 antigen and ankylosing spondylitis. At least 90% of patients with ankylosing spondylitis are B27 positive. The association of B27 with similar spondylitides, such as Reiter's syndrome, the spondylitis of psoriatic arthritis, or the spondylitis of inflammatory bowel disease, is lower. Isolated peripheral joint arthritis in the latter two conditions is not associated with HLA-B27, however. HLA-B27 is present in 6 to 8% of the American caucasian population and 2 to 4% of the American black population. Approximately 20% of B27-positive individuals are likely to develop clinically significant ankylosing spondylitis.

The test for determining the presence of B27 antigen is expensive, and arguments have been made against the utility of the test for differential diagnosis of ankylosing spondylitis, since the disease could still be present in a B27-negative patient and the majority of B27-positive individuals do not develop the disease. It should not be used as a routine screening test, but may be helpful in confirming a clinical suspicion when the diagnosis is not clear. Examples include the presence of apparently inflammatory low back pain in young patients before sacroiliac roentgenographic changes are present, incomplete Reiter's syndrome in patients who lack the extraarticular signs of the disease, or juvenile arthritis patients who are seronegative for rheumatoid factor and antinuclear antibody when a prognostic indicator is desired.

Another clinical situation in which the determination of HLA type may be useful applies to rheumatoid arthritis.

Table 167.5
HLA-associated Rheumatic Diseases

Disease	HLA antigen
Ankylosing spondylitis	B27
Reiter's syndrome	B27
Reactive arthritis *(Yersinia, Salmonella, Shigella)*	B27
Psoriatic arthritis	
Peripheral	Bw38, Bw39
Spondylitis	B27
Inflammatory bowel disease	
Spondylitis	B27
Rheumatoid arthritis (adult)	DR4, DR1
Juvenile arthritis	
Seropositive, polyarticular	DR4
Pauciarticular	DR5, Dw5, DRw8, Dw52, DPw2, DRw6
Spondylitis	B27
Still's disease	?Bw35, DR4
Systemic lupus erythematosus	DR3, DR2, DQw1
With SS-A (Ro)	DR3
With C_2 deficiency	DR2(Dw2), A25, B18
Subacute cutaneous lupus erythematosus	DR3
Drug-induced lupus erythematosus	DR4
Sjögren's syndrome	
Primary	DR2, DR3, DRw52
With rheumatoid arthritis	DR4, DRw52, DRw53
With systemic lupus erythematosus	DR3, DQw1/DQw2, DRw52
Polymyositis	
Caucasians	DR3
Blacks	DRw6
Jo-1 positive	DR3, DRw6
Childhood	B8, DR3
Scleroderma	DR5
CREST	DR3
Anticentromere positive	DR1

Whereas the DR4 antigen has been shown to be associated with seropositive rheumatoid arthritis, the incidence of HLA-DR3 positivity has been reported to be very high in rheumatoid arthritis patients who develop proteinuria and thrombocytopenia with gold therapy, and proteinuria with penicillamine therapy. The presence of the DR3 antigen in a patient who has developed proteinuria due to gold therapy would suggest that penicillamine therapy might also cause proteinuria.

References

Arnett FC. Immunogenetics and arthritis. In: McCarty DJ, ed. Arthritis and allied conditions. 10th ed. Philadelphia: Lea & Febiger, 1985;405–15.

Calin A. HLA-B27: to type or not to type? Ann Intern Med 1980;92:208–11.

Goldstein R, Arnett FC. The genetics of rheumatic diseases in man. Rheum Dis Clin N Am 1987;13:487–510.

Joysey VC. Advances in tissue typing. In: Lachman PJ, Peters DK, eds. Clinical aspects of immunology. 4th ed. Oxford: Blackwell Scientific Publications 1982;462–75.

Moller G, ed. HLA and disease susceptibility. Immunol Rev 1983; 70:1–218.

Schiffenbauer J, Schwartz BD. The HLA complex and its relationship to rheumatic diseases. Rheum Dis Clin N Am 1987;13:463–85.

Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High association of an HLA antigen, W27 with ankylosing spondylitis. N Engl J Med 1973;288:704–6.

Schwartz BD. The human major histocompatibility HLA complex. In: Stites DP, Stobo JD, Fudenberg HH, Wells JV, eds. Basic and clinical immunology. 6th ed. Norwalk and Los Altos: Appleton and Lange, 1987;50–64.

Trowsdale J, Young JA, Kelly AP, et al. Structure, sequence and polymorphism in the HLA-D region. Immunol Rev 1985;85:5–43.

Winchester RJ. The major histocompatibility complex. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, eds. Textbook of rheumatology. 3rd ed. Philadelphia: W.B. Saunders, 1989;101–137.

Wooley PH, Griffin J, Panayi GS, Batchelor JR, Welsh KI, Gibson TJ. HLA-DR antigens and toxic reactions to sodium aurothiomalate and D-penicillamine in patients with rheumatoid arthritis. N Engl J Med 1980;303:300–32.